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AUSTRALIA

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BIOMOLECULAR RESEARCH INSTITUTE LIMITED

PROVISIONAL SPECIFICATION

Invention Title:

Beta-amyloid peptide inhibitors

The invention is described in the following statement:

Aβ has been shown to bind copper and iron in stoichiometric amounts with the associated formation of reactive oxygen species such as peroxides and hydroxide radicals, possible sources of the neurotoxicity (Bush et al., 1998). While the formation of peroxide in post-mortem samples of Alzheimer's diseased brain was observed, there was little peroxide formation in control tissue (Cherny et al., 1998). The activity observed in the samples of Alzheimer's disease brain was abolished when treated with certain chelators (Cherny et al., 1998). The formation of reactive oxygen species was accompanied by the reduction in the valence state of the metal ie Cu(II) to Cu(I) and Fe(III) to Fe(II) (Atwood et al., 1998a). Reactive oxygen species can also lead to radical formation on the Aβ peptide and over time a consequent covalent cross-linking of the Aβ peptides (Bush et al., 1998). In addition a number of metal ions including Zn, Ni and Cu have been shown to induce aggregation of Aβ (Atwood et al., 1998b). When brain tissue (both control and Alzheimer's disease affected) were treated with chelators specific for zinc and copper there was greatly enhanced solubilisation of Aβ (an increase of up to 700%), suggesting that these metals play a role in the assembly of the Aβ deposits (Cherny et al., 1998).

Histidine residues have been implicated in the binding of metal ions to Aβ peptides. For instance rat Aβ1-40 (in which His13 is mutated to Arg) does not aggregate nor does Aβ1-40 treated with diethyl pyrocarbonate, which binds to the imidazole nitrogen of histidine (Atwood et al., 1998).

Inhibition of zinc and copper binding to the Aβ peptide is likely to have significant therapeutic value in the treatment of Alzheimer's disease.

SUMMARY OF THE INVENTION

The present inventors have now found that zinc and copper bind predominantly to a region in the N-terminal loop of Aβ which includes a cluster of histidine residues. This finding provides the basis for the rational design or selection of inhibitors to zinc, copper and iron binding to Aβ.

Accordingly, in a first aspect the present invention provides a compound which interacts with the β-amyloid peptide such that the N-terminal loop of the peptide (residies 1 -15) is blocked or destabilised thereby inhibiting the binding of one or more metal ions to at least one histidine residue within the N-terminal loop.

The term "targeting moiety" as used herein refers to a functional group that will specifically interact with the β-amyloid peptide. That is, the inhibitor compound includes or is conjugated to a targeting moiety that will specifically bind or associate with the β-amyloid peptide. Suitable targeting moieties include, but are not limited to polypeptides, nucleic acids, carbohydrates, lipids, β-amyloid ligands, antibodies, dyes and the like. In a preferred embodiment the targeting moiety has a hydrophobic region which interacts with the tail of the β-amyloid peptide. The targeting moiety may include, for example, a fatty acid molecule.

In a preferred embodiment, the targeting moiety targets the compound to the site defined by residues 15-21 on the β-amyloid peptide. The targetting moiety may be a peptide which has or includes a sequence which corresponds to that of residues 15-21 on the β-amyloid peptide.

In a second aspect the present invention provides a method of selecting, or designing, a compound which inhibits the binding of metal ions to the N-terminal loop of the β-amyloid peptide which method includes

(i) selecting or designing a compound which has a conformation and polarity such that it binds to at least one, more preferably at least two and more preferably three amino acids in the N-terminal loop selected from the group consisting of His6, His 13 and His14; and

(ii) testing the compound for the ability to inhibit binding of metal ions to the N-terminal loop.

In a preferred embodiment of the second aspect, the compound has a conformation or polarity such that it also binds to at least one amino acid in the N-terminal loop selected from the group consisting of Asp7, Tyr10, and Glu11.

In a third aspect the present invention provides a compound which inhibits the binding of metal ions to the N-terminal loop of the β-amyloid peptide, wherein the compound is obtained by a method according to the second aspect of the present invention.

In a fourth aspect the present invention provides a pharmaceutical composition including a compound according to the first or third aspects of the present invention.

In a fifth aspect the present invention provides a method of inhibiting the binding of one or more metal ions to the β-amyloid peptide, or inhibiting the aggregation of β-amyloid peptides, which method includes exposing the

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Representation of the β-amyloid peptide showing structured turn in the region of residues 15-21.

Figure 2. A model of Zn bound to the three histidine residues of Aβ1-40.

Figure 3. NMR spectrum showing the effect of Zn^{2+} binding to βA1-28.

Figure 4. NMR spectrum showing the effect of Cu^{2+} binding to βA1-28.

Figure 5. NMR spectrum showing the effect of addition of Cu^{2+} and cobalt complex to βA1-28.

Figure 6. NMR spectrum showing the binding of the cobalt complex to βA1-28.

Figure 7. Western Blot showing results of brain tissue assays testing the ability of a range of metal compounds to solublilise Aβ deposits.

Figure 8. A model of the cobalt-corrin ring complex bound to Aβ1-40.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have developed 3D structural information concerning the N-terminal region of the β-amyloid peptide and have identified a cluster of three histidine residues which constitute a binding site for metal ions. This information provides a rational basis for the development of compounds which inhibit the binding of metal ions to the N-terminal loop of the β-amyloid peptide. Such inhibitors have the potential to inhibit aggregation of β-amyloid peptides and to reduce metal induced neurotoxicity. Accordingly, these inhibitors are likely to have therapeutic value in the treatment of diseases such as Alzheimer's disease.

In summary, the general principles of drug design can be applied by persons skilled in the art to produce compounds which preferably bind to at

In these formulae:

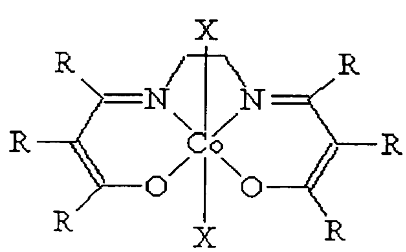
(i) a, b, c, d and e are non-leaving groups, preferably chelating groups including Schiff bases, porphyrin rings, macrocycles (these multidentate ligands could have a variety of donor atoms), polyamino-carboxylates, heterocyclic aromatics such as 2,2' bipyridine and 1,10-phenanthrolene, peptides, nucleobases, chelating ligands where one of the donor atoms is a phosphine. In addition it is possible that one or more of the non-leaving groups could be stable monodentate ligands such as cyanide or an organic group (eg methyl group).

(ii) M is a metal.

(iii) w, x, y and z are leaving groups (ie those groups which will be replaced by histidine and possibly other residues when the metal complex reacts with the β-amyloid peptide) and include halogens, amines, ammonia, pyridyls, imidazoles, nucleobase, peptides, H_2O/OH,carboxylic acids, phosphates, sulfates, nitrate, triflate, alkoxides.

Those skilled in the art will recognise that the appropriate combination of non-leaving and leaving groups will be dependent on the metal.

Metal complexes of the which have the potential to bind to histidine residues are described in WO 97/21431 and WO 96/18402, the entire contents of which are incorporated herein by reference. Other examples of complexes which may act as inhibitors are as follows:



paramagnetic metals. Assays are available for measuring Cu/Fe reduction, hydrogen peroxide, hydroxyl radical generation, and a carbonyl assay, all of which assess the redox capacity of Aβ in the presence of Cu and Fe. *Ex vivo* assays using post-mortem brain tissue may also be performed. These include measuring the amount of Aβ which is solubilised and extracted in the presence of drug, and determining the quantity of peroxide formed in post-mortem brain tissue as compared with control tissue.

In a preferred embodiment of the present invention, the inhibitor compound is conjugated to a targeting moiety.

The term "targeting moiety" as used herein refers to a functional group that will specifically interact with the β-amyloid peptide. That is, the inhibitor compound is covalently linked to a targeting moiety that will specifically bind or associate with the β-amyloid peptide. Suitable targeting moieties include, but are not limited to polypeptides, nucleic acids, carbohydrates, lipids, β-amyloid ligands, antibodies and the like. In a preferred embodiment the targeting moiety has a hydrophobic region which interacts with the tail of the β-amyloid peptide. The targeting moiety may include, for example, a fatty acid molecule.

In a further preferred embodiment, the inhibitor -targeting moiety complex is able to pass the blood-brain barrier.

The compounds of the present invention may be formulated into pharmaceutical compositions, and administered in therapeutically effective doses. By "therapeutically effective dose" is meant a dose which results in the inhibition of natural binding of metal ions to the N-terminal loop of the β-amyloid peptide. The appropriate dose will be ascertainable by one skilled in the art using known techniques.

The pharmaceutical compositions may be administered in a number of ways, including, but not limited to, orally, subcutaneously, intravenously, intraperitoneally and intranasally.

The present invention is further described below with reference to the following, non-limiting examples.

Metal Binding Studies

Metal binding studies were performed by titrating concentrated metal solutions (30 mM $CuCl_2$, $ZnCl_2$ in water) into the peptide solutions described above. The displacement of bound Cu^{2+} from Aβ1-28 by the Co(III) Schiff-base was performed by adding one equivalent of Cu^{2+} to Aβ1-28 followed by one equivalent of Co(III) Schiff-base.

Brain Tissue Assays

Tissue selection: Post-mortem tissues, stored at -80°C, were obtained from the NH&MRC supported Brain Bank at the University of Melbourne, together with accompanying histopathological and clinical data. AD was assessed according to CERAD criteria (Mirra et al, 1991). In order to examine the chemical architecture of the Aβ deposition that it is observed in non-AD aged brain, Aβ immunohistochemistry was used to select age-matched control (AC) cases that did not reach CERAD criteria, and in which amyloid deposition, if present, was detectable only in the form of diffuse plaques, but not neuritic plaques.

Selection of compounds: Compounds were dissolved in DMSO and diluted in a PBS mixture. Insoluble material was removed.

Sample preparation: The cortical meninges were removed and gray matter (0.5 g) was homogenised using a DIAX 900 homogeniser (Heidolph & Co, Kelheim, Germany) for 3 x 30s periods at full speed, with a 30s rest between strokes, in 3 ml of ice-cold phosphate-buffered saline ("PBS"), pH 7.4, containing a mixture of protease inhibitors (BioRad, Hercules, CA), with the exception of EDTA, or in the presence of either various chelators or metal ions prepared in PBS. To obtain the PBS-extractable fraction, the homogenate was centrifuged at 100,000 x g for 30 min, the supernatant removed and divided into 1 ml aliquots. Protein within a 1ml supernatant sample was precipitated using 1:5 ice-cold 10% trichloracetic acid (TCA), and pelleted by centrifugation at 10,000 x g for 20 mins. The pellet was prepared for PAGE by boiling for 10 min in Tris-tricine SDS-sample buffer containing 8% SDS, 10% mercaptoethanol and 8M urea. Total Aβ in the cortical samples was obtained by homogenizing in 1 ml PBS and boiling in sample buffer as above.

Aβ1-40 chemical shifts with random coil chemical shifts and the lack of NOE connectivities in the NOESY spectra indicate that both peptides are mostly in conformational exchange. However there are some medium range NOE connectivities ($1 < |i\text{-}j| < 5$) observed in region of residues 16-21 of the peptide (KLVFFA) suggesting that this region of the peptide has a structured turn (Figure 1). This region of the peptide has previously been shown to be very important in defining the aggregation properties of Aβ (Hilbich et al. 1992), with the substitution of hydrophillic residues into this region resulting in altered aggregation properties including reduced β-sheet content. In addition several groups have described short peptides or slight variants thereof corresponding to this region which have the ability to bind to Aβ and inhibit the formation of fibrils (Findeis et al. 1999 & Tjernberg et al. 1999). This evidence implies that this "structured" section of Aβ is important in the formation of amyloid fibrils.

Metal Binding Studies

To determine the metal-binding site of Aβ1-40, Zn^{2+} was titrated into a solution of Aβ1-40 in SDS-micelles at pH 6.5. Peaks due to the C2H protons of the imidazole rings of His6, 13 and 14 broadened out such that they were no longer visible on the addition of a small amount of Zn solution (~ 25% of one mol. equivalents.). The addition of extra Zn (up to two mol. equivalents) did not change the spectrum, but when the pH of the solution was raised to 7.4 three broad overlapping peaks due to the C2H protons of the imidazole rings of His6,13 and 14 became visible, these peaks did not sharpen significantly even upon the addition of a large excess of Zn (> 150 mol. equivalents). There appears to be no significant differences in the rest of the spectrum between the Zn-bound and free forms of Aβ1-40 suggesting that there are no significant conformational changes upon metal binding. These results indicate that all three histidine residues of Aβ1-40 are involved in Zn binding. Figure 2 shows a model of Zn bound to the three histidine residues of Aβ1-40.

To determine the metal-binding site of Aβ1-40 and Aβ1-28 in aqueous solution, Zn^{2+} and Cu^{2+} were titrated into solutions of Aβ1-40 and Aβ1-28 pH 6.9. All reactions were accompanied by significant precipitation. The NMR spectrum of the peptide-metal complex that remained in solution showed peaks due to the C2H and C4H protons of the imidazole rings of His6, 13 and

Discussion

When copper and iron binds to Aβ reactive oxygen species such as peroxide and superoxide are produced. When copper and zinc bind Aβ both induce aggregation and copper binding is inhibited by zinc, suggesting a similar binding site. Zinc and presumably copper bind to the histidine residues of Aβ, a molecule which prevents zinc and copper binding to the histidine residues has the potential to inhibit aggregation and prevent metal induced neurotoxicity.

Compounds of the sort described herein have the potential to bind to histidine residues and therefore prevent zinc and copper binding, and so may have therapeutic value. A model of a Cobalt-corrin ring complex bound to Aβ1-40 is shown in Figure 8.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty third day of July 1999

BIOMOLECULAR RESEARCH INSTITUTE LIMITED
Patent Attorneys for the Applicant:

F B RICE & CO

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Figure 1

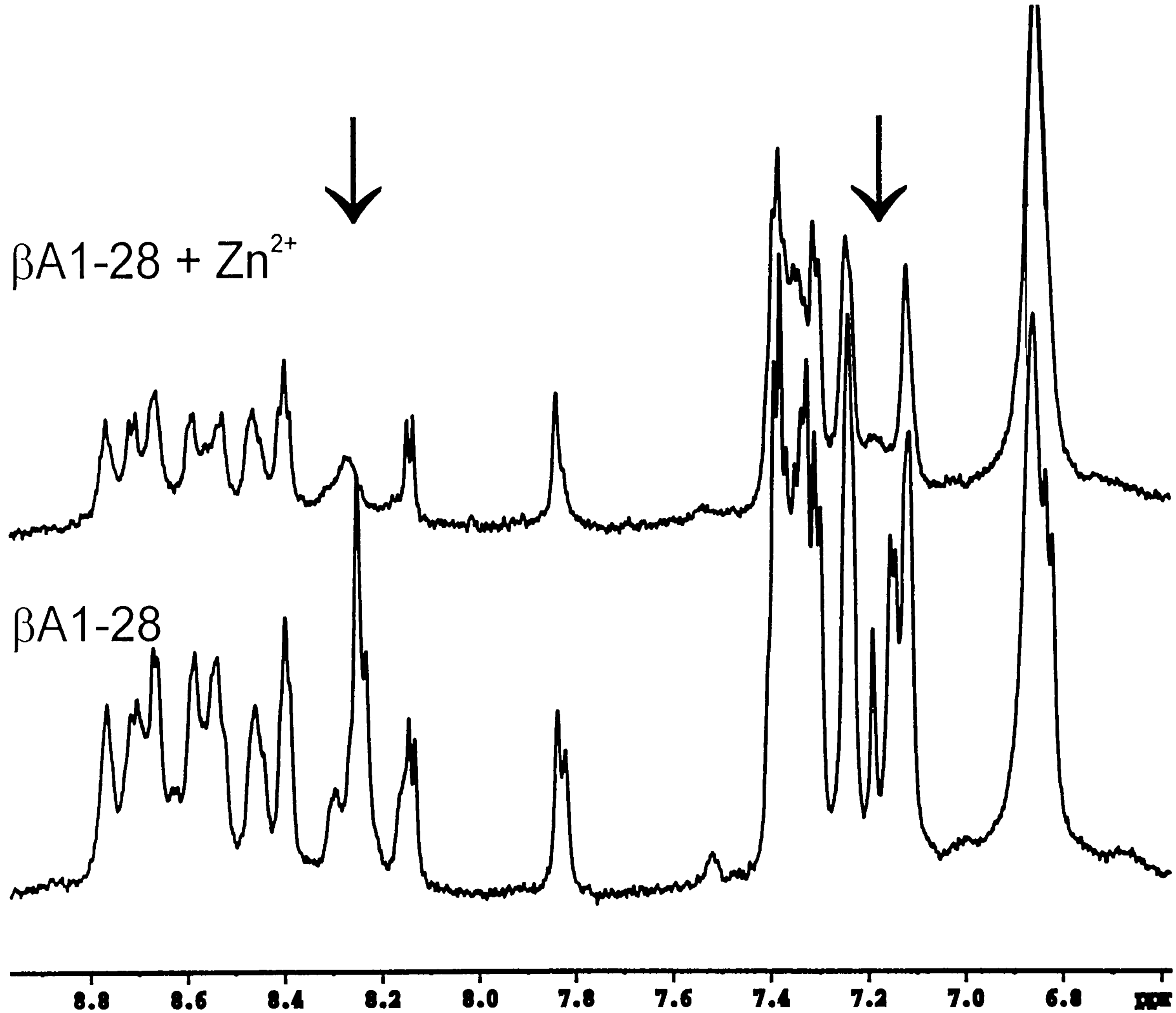


Figure 3

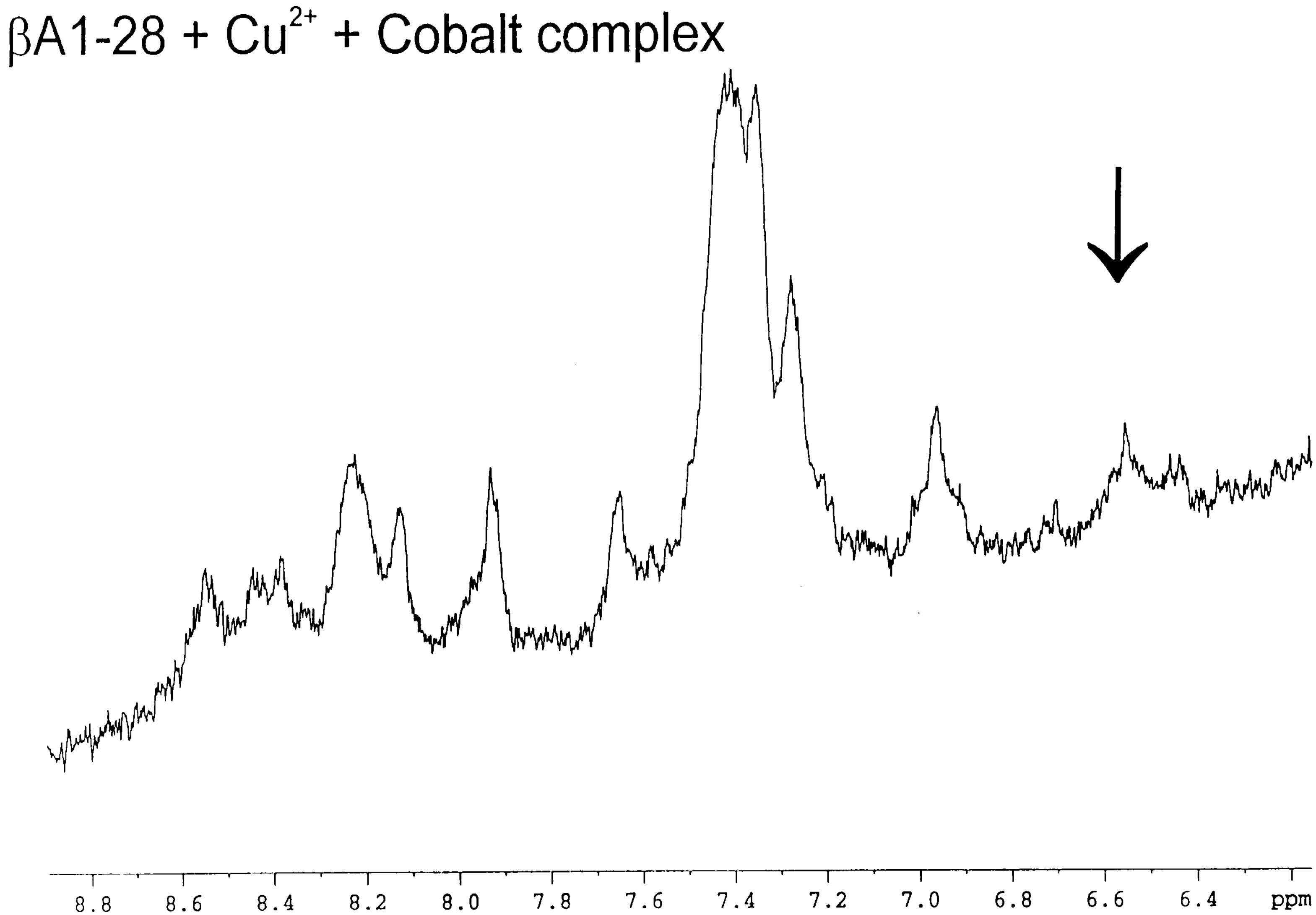
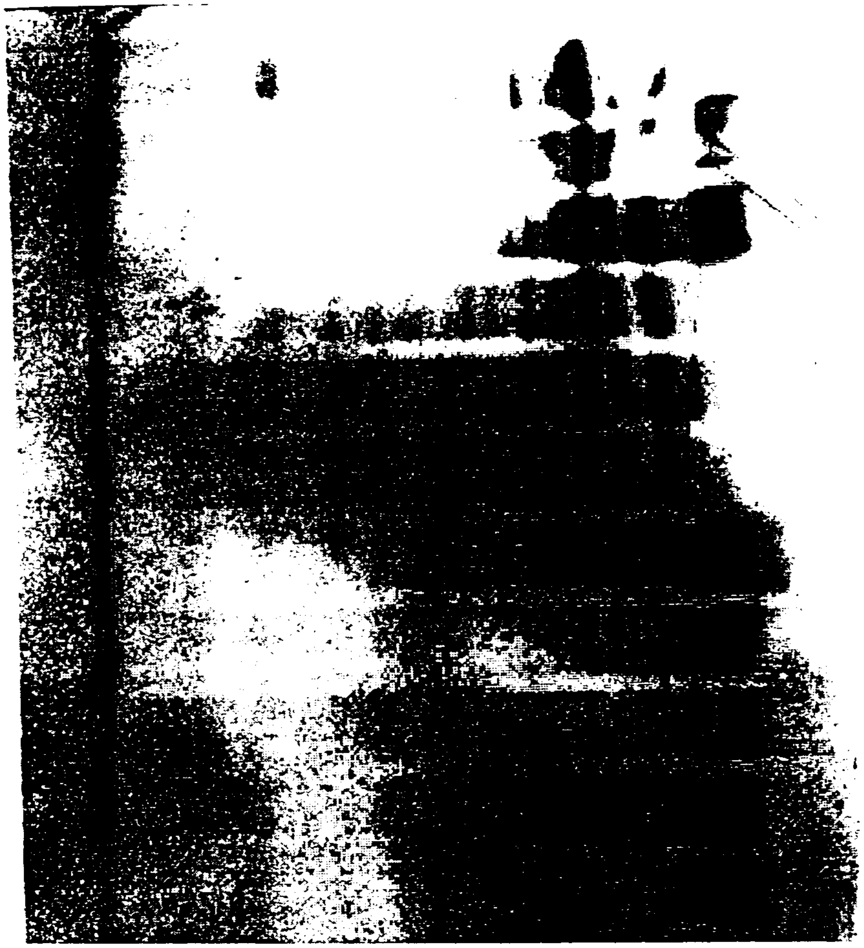

βA1-28 + Cu2+ + Cobalt complex
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
ppm

Figure 5



PBS

KJB 001

KJB 002

KJB 003

KJB 004

KJB 005

KJB 006

KJB 007

KJB 008

KJB 010

BRI 6805

Figure 7